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An LCMS Based Assay for Detection and Quantification of Bacterial Cell Wall Intermediates

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An LCMS Based assay for detection and quantification of bacterial cell wall intermediates.

A Project

Submitted

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By

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Dedicated to

My Family

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Abstract:

The main objective of my project is to determine the bacterial cell wall intermediates in E.coli and develop an **LCMS** method that Phosphomycin is a PEP analogue that irreversibly inhibits UDP-GluNAc-enolpyruvate transferase (MurA enzyme), which prevents the formation of N-acetyl muramic acid, which is an essential element in the Peptidoglycan cell wall. Antibacterial agents have a tremendous impact on human health. Most of the antibacterial agents, including beta lactams target the bacterial cell wall biosynthesis.[1] The pathway for Escherichia coli is shown in page 19. Among the most widely used antibiotics, beta lactams inhibit the penicillin binding protein (PBPs) [1] which block the cross linking reactions of cell wall biosynthesis. A Liquid chromatography-tandem mass spectrometry provides a new technology to detect and quantitation of cell wall intermediates.

Introduction

Tandem mass spectrometry (MS/MS) coupled with HPLC (high pressure liquid chromatography) is the analytical technique of choice for most assays used during new drug discovery. Some applications are:

- **Biotechnology:** Mainly used in analysis of proteins, peptides, oligonucleotides
- **Pharmaceutical:** Mainly used in drug discovery, pharmacokinetics, drug metabolism
- **Clinical Biochemistry and Toxicology:** neonatal screening, haemoglobin analysis, drug testing.

MS/MS used with or without chromatographic separation offers the advantage of 1) analytical sensitivity and 2) selectivity for drug and drug metabolite analysis. These advantages are achieved through reduction in interferences from other sample components. This allows the development of analytical methods for complex mixtures that are fast, require less stringent sample preparation, less chromatographic separation and therefore consumes much less solvent, allowing higher sample throughput. Single stage mass spectrometry measures the molecular mass of a compound and/or its fragments. MS/MS consists of two or more mass spectrometer analyzers all in a single instrument. More popular tandem mass spectrometers include those of the quadrupole-quadrupole type (also known as Triple Quadrupole instruments), or the hybrid types**:** including magnetic sector/quadrupole, and more recently, the quadrupole/time-of-flight (Q-TOF) geometries.

Tandem mass spectrometer consists of mainly 3 parts:

1) Ionization source,

2) Analyzer

3) Detector**.**

A sample introduced into the ionization source becomes ionized. This makes the sample components easier to manipulate. The ions are removed into the analyzer where they are separated according to their mass -to-charge ratios (m/z). The separated ions are then fragmented. The fragments are detected and the signal created by the detected fragment ions is sent to a data system where the m/z ratios are stored together with their relative abundance.[15]

Hybrid triple quadrupole/Linear Ion Trap technology provides high sensitivity. By using this quadrupole scanning functionality with sensitive linear ion trap scans we can reduce the analysis time and get more information for every experiment. This linear ion Trap technology of 3200 Q TRAP system is used to identify and quantify components from complex samples in a single run.We need some specification to run this 3200 Q TRAP LCMS software on a personal computer. We have to maintain the Mass range m/z 5-1,700 in quad mode 50-1,700 in linear ion trap mode. Scan speed is upto2400 amu/sec in quad mode and upto4000 amu/sec in linear ion trap mode [16]. This instrument contains different types of scan modes which are traditionally not available in a single detector. These modes allow quantification, confirmation and identification with a single detector**.** The most common detectors used are photomultiplier, the electron multiplier and the micro-channel plate detectors.

Materials:

Chemicals:

E.coli, UDP-Glucose, UDP-N acetyl glucose, Solvent A, Solvent B, Solvent C, Ion pair reagent, Acetone, water, Liquid nitrogen, LB Broth media.

Solvent A: water +0.1N HCOOH

Solvent B: 70% C + 30% A.

Solvent C: Aceto nitrile + 0.1N HCOOH.

Ion pair reagent: Dimethyl Hexyl Amine 160 mM P^H at 3.

Instruments: 3200 Q TRAP ION CHARGE LCMS, RT 6000 Refrigerated Centrifuge.

Methodology:

Scan types: Multiple reactions monitoring (**MRM**)

Enhanced Product Ion scans. (**EPI**)

Precursor Ion scans**.** (**PI)**

MRM METHOD:

Multiple Reaction monitoring (MRM)*:* It Involves both analyzers such that only ions of selected molecular weight are allowed to pass through the first analyzer and only specifically selected fragments arising from these are measured by the second analyzer. When a sample is introduced into Q1 it passes into Q2 there it undergoes cell collision and fragmentation takes place and these fragments are analyzed by the Q3. This method is widely used in drug testing in blood and urine samples. It is also very sensitive and specific.

Q1	Q2	Q3

Q1= Parent mass of the compound.

Q2= Cell collision takes place and fragmentation takes place.

Q3= Scan the fragment mass.

Enhanced Product ion scan: Here we scan the product ions in Q3 (MS2), where as the mass in Q1 (MS1) is static. So we should know the compound mass.

Precursor Ion scan: Here we scan the precursors in Q1 (MS1), where as the mass in Q3 (MS2) is static. So we need to know the fragment mass of the compound.

In general we use MRM method.

Before using this instrument we need to know some parameters involved in Quadrupole mode.

Declustering potential (DP)

Entrance potential (EP)

Collision Cell Entrance Potential (CEP)

Growth and preparation of bacterial extracts:

A saturated culture of E.coli was grown in an incubator shaker overnight at 37□C. This culture was placed in a rotary shaker and incubated with good agitation at 37□C. When the culture reached OD value to 0.8 at 600nm, a 1/3rd ml portion of culture was placed into sterilized 250 ml flask.[1] from figure 9 flask on left contains sterile medium and flask on right contains medium inoculated with E. coli bacteria the day before. The turbidity in second flask due to bacterial cells.[16]

Procedure:

To identify the intermediates of the cell pathway of E.coli, we use E.coli bacterial extract. Here we are using an ion pair reagent (n, n dimethyl hexylamine) [15] because UDP has high negative charges in the structure. This ion pair reagent has positive charges. Because of UDP have high negative charge ions we are running in negative mode i.e. polarity is negative. The main use of Ion pair reagent is ionic samples form an ion pair with ion pair reagents in the mobile phase to become electrically neutral. By using this ion pair reagent we can enhance peak shape and retention time. In this E.coli extract run we have seen only UDP-GluNAc and UDP-MurNAc peaks. Tandem Mass Spectrometry also know as Mass spectrometry, involves multiple steps of mass spectrometry selection, with some form of fragmentation occurring between the stages.

MS Optimization:

To remove salts prior to MS optimization, a 50ul of sample is taken and loaded into 200mg of C18 silica in a 1-ml syringe prepared by first washing with methanol and then equilibrated with solvent 'A'. After some time the resin was washed with solvent 'B'. We have to wash with solvent 'C' also in order to elute salts from the sample.[1]

Selection of Internal standard:

- For internal standard, we have to select either ^{13}C or ^{14}C but 14C is a radioactive, we are using ^{13}C.
- ^{13}C UDP-Glucose is not naturally abundant in nature. In nature ^{12}C is 98.8% abundant where as ^{13}C is around 1.1%.
- ^{13}CUDP-Glucose--- internal standard.

We are using ^{13}CUDP-Glucose as internal standard because UDP-Glucose and ^{13}CUDP-Glucose are structurally similar only changing in the molecular weight. Before using the instrument wash

every time syringe with methanol and run the blank with methanol to find out that intensity of peak is looking better or not. Next in order to calibrate the instrument take the internal standard in syringe and run the mass spectrometer to find out it shows 567.302 or not. Because it is a stock solution it will show Molecular weight of 567.302

Internal standard is a disodium salt when we add ion pair reagent disodium salts is eluted. We have to tune the internal standard. By tuning ^{13}CUDP-Glucose peaks were obtained.

Quantitative optimization by infusion method:

Infusion is the continuous flow of a sample at low flow rate into the source using a syringe pump. Typically flow rates are 5 to 25uL/min. The infusion method is mainly useful for pure standard that can be used for tuning process.

In this we have to tune the sample what we want exactly i.e. 567.302 For this we have to select Mass+ syringe type option and we have to run the sample which is ^{13}CUDP-Glucose by taking negative polarity. Here fragments also we can analyze. We can select a mass range between 566.5 to 568.0 and then select finish automatically it shows the molecular weight of 567.302

Growth and preparation of bacterial extracts for analysis of antibiotic effects:

A saturated culture of E.coli was grown in an incubator shaker overnight at 37°C. Cells were harvested by centrifugation and resuspended to the desired optical density (O.D 0.5 at 600nm). This culture was placed in a flask and incubated with good agitation at 37°C. when the culture reached to O.D =0.5 3/4th of the portions were placed in 250 ml sterilized culture flask. Antibiotics (Ampicilin, cycloserine and phosphomycin) were added to 8x MIC (MIC =8ug/ml for Ampicilin and Cycloserine, for phosphomycin MIC=4ug/ml) in individual flask plus control flask without antibiotic. We need to choose the antibiotic based on bacteria i.e. E.coli which is gram negative bacteria.

Growth inhibition was observed with in 15min. After 30 min, the cultures were rapidly cooled in ice bath, 4 samples of 10ml were taken into each flask to ice-cold 15ml centrifuge tubes, and cells were pelleted by centrifuge at 3000rpm for 10 min. cell pellets were treated 80%acetone and shake well and again centrifuge for 10min.[1] Supernatants were collected in fresh micro centrifuge tubes. Samples were taken and run the LCMS. From the figure 10 we can plot a graph between time vs optical density for the confirmation of bacterial growth is good or not. The solid diamond represents Control, Solid Square represents Cycloserine, solid triangle represents Ampicilin and solid x represents Phosphomycin.

Results and Discussions:

Form the Figure 11 we can conclude that, a control sample treated with Ampicilin and Cycloserine antibiotics expected to have small effects on UDP-GlcNAc, UDP-MurNAc. The results from this experiment are illustrated in figure 11. Phosphomycin is a PEP analogue that irreversibly inhibits UDP-GluNAc-enolpyruvate transferase which is also called as (MurA enzyme), which prevents the formation of N-acetyl muramic acid, which is an essential element in the Peptidoglycan cell wall.

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Figure 1:

E.coli Pathway: **UDP-GlcNAc**

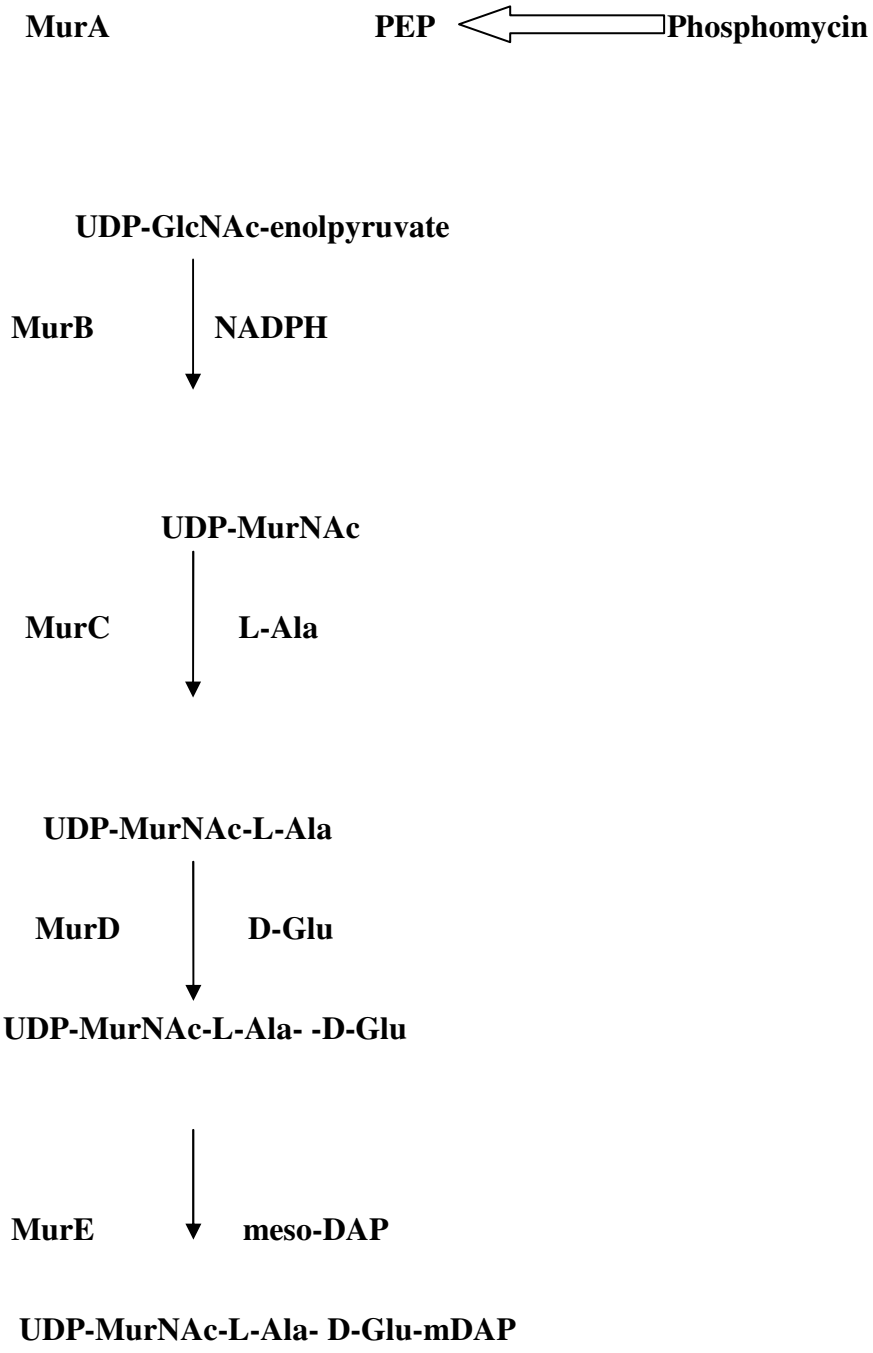

MurA
PEP
Phosphomycin
UDP-GlcNAc-enolpyruvate
MurB
NADPH
UDP-MurNAc
MurC
L-Ala
UDP-MurNAc-L-Ala
MurD
D-Glu
UDP-MurNAc-L-Ala- -D-Glu
MurE
meso-DAP
UDP-MurNAc-L-Ala- D-Glu-mDAP

Figure 2:

Structure of UDP-GlcNAc:

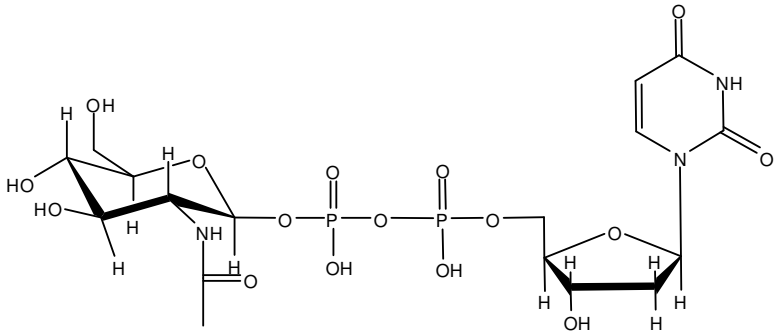


Figure 3:

Structure of UDP-GlcNAc-enolpyruvate:

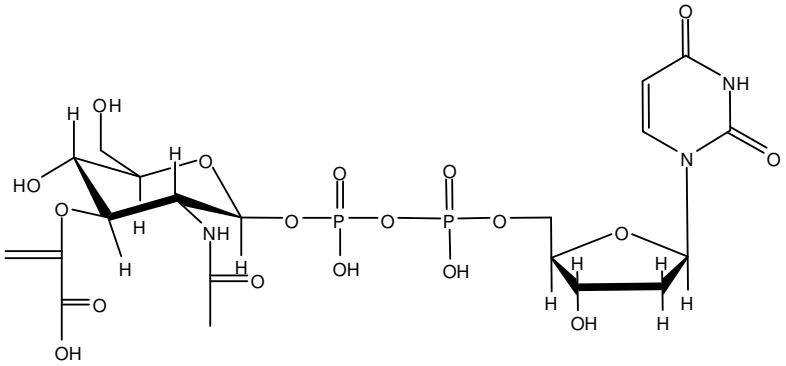


Figure 4:

Structure of UDP-MurNac :

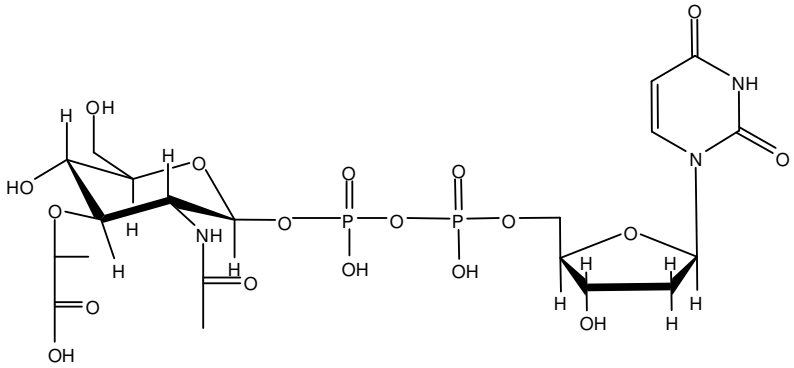


Figure 5:

Structure of UDP-MurNac-L-Ala:

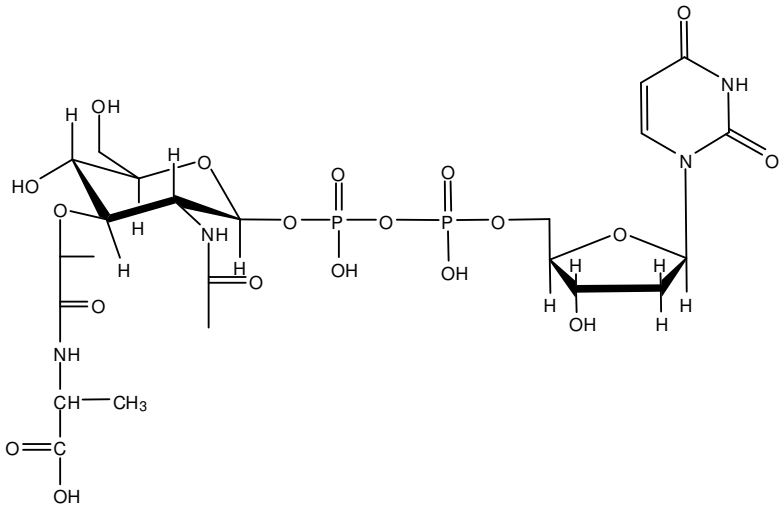


Figure 6:

Structure of UDP-MurNac-L-Ala-□-D-Glu:

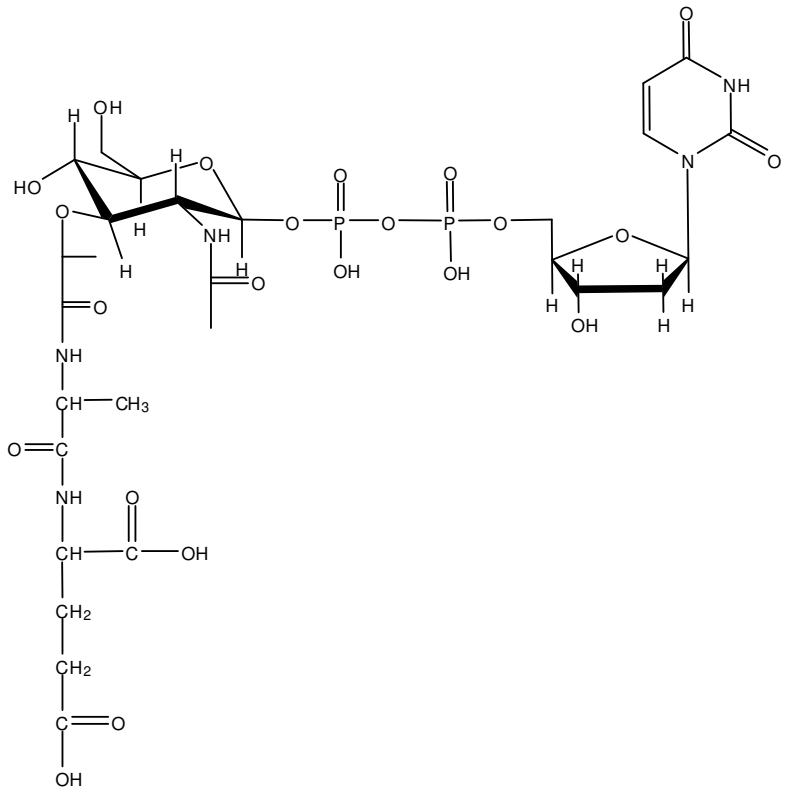


Figure 7:

Structure of UDP-MurNac-L-Ala- □-D-Glu-Meso-diamino-pimelic acid:

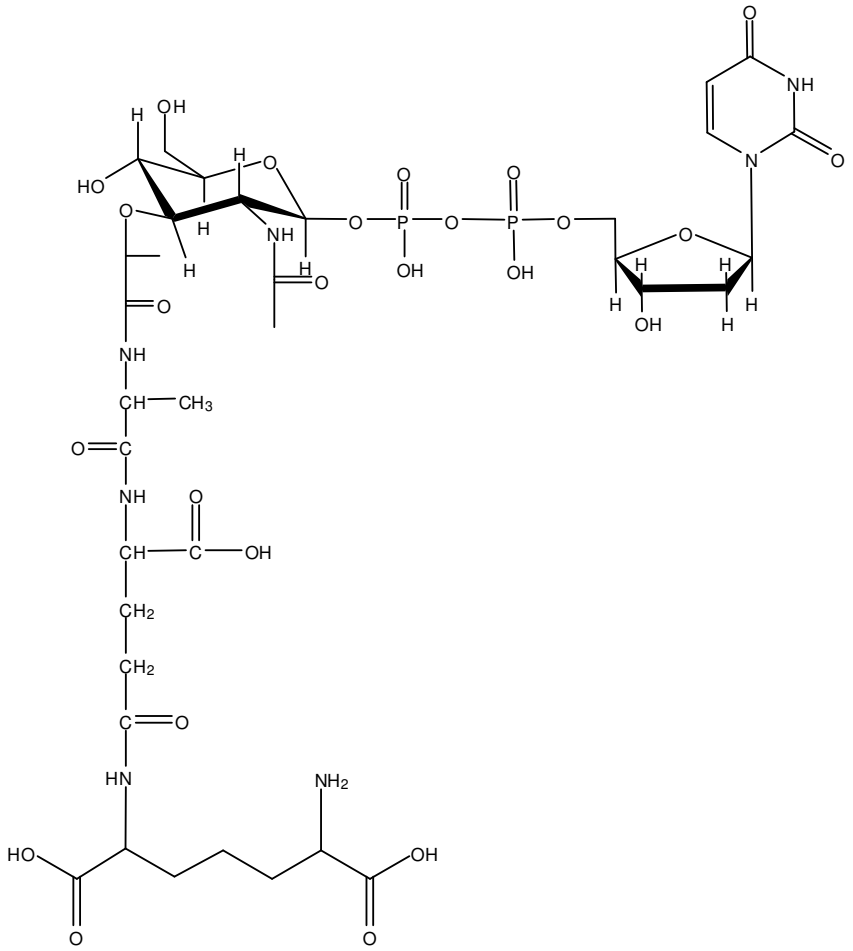


Figure: 8

3200 QTRAP LCMS:

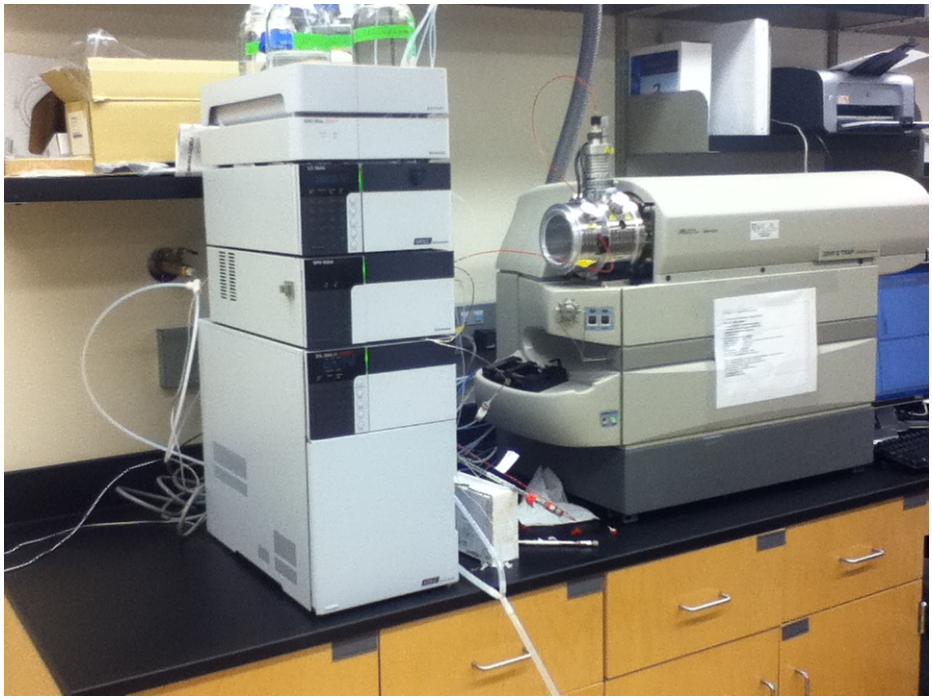


Figure 9:

Sterile medium vs E.coli bacterial medium

Table 1:

O.D. Values for different antibiotic samples:

Time	Control	Cycloserine	Ampicilin	Phosphomycin
0	0.481			
5	0.495	0.495	0.495	0.495
10	0.535	0.538	0.53	0.534
15	0.537	0.553	0.541	0.557
20	0.582	0.568	0.555	0.576
25	0.651	0.502	0.573	0.533
30	0.725	0.488	0.603	0.527
35	0.757		0.591	0.459
40	0.796		0.545	

Figure 10:

E.coli Growth Curve:

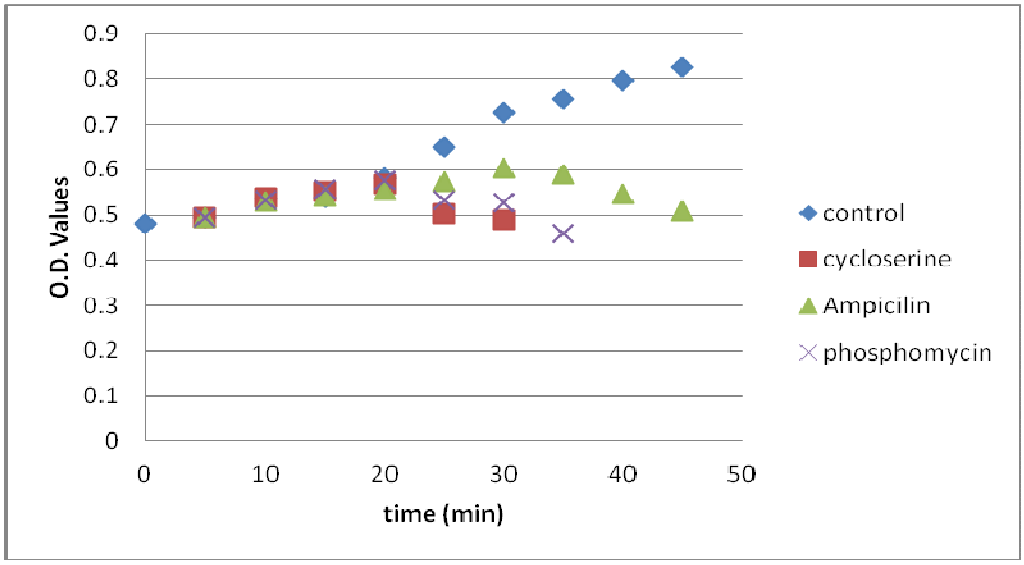


Table 2:

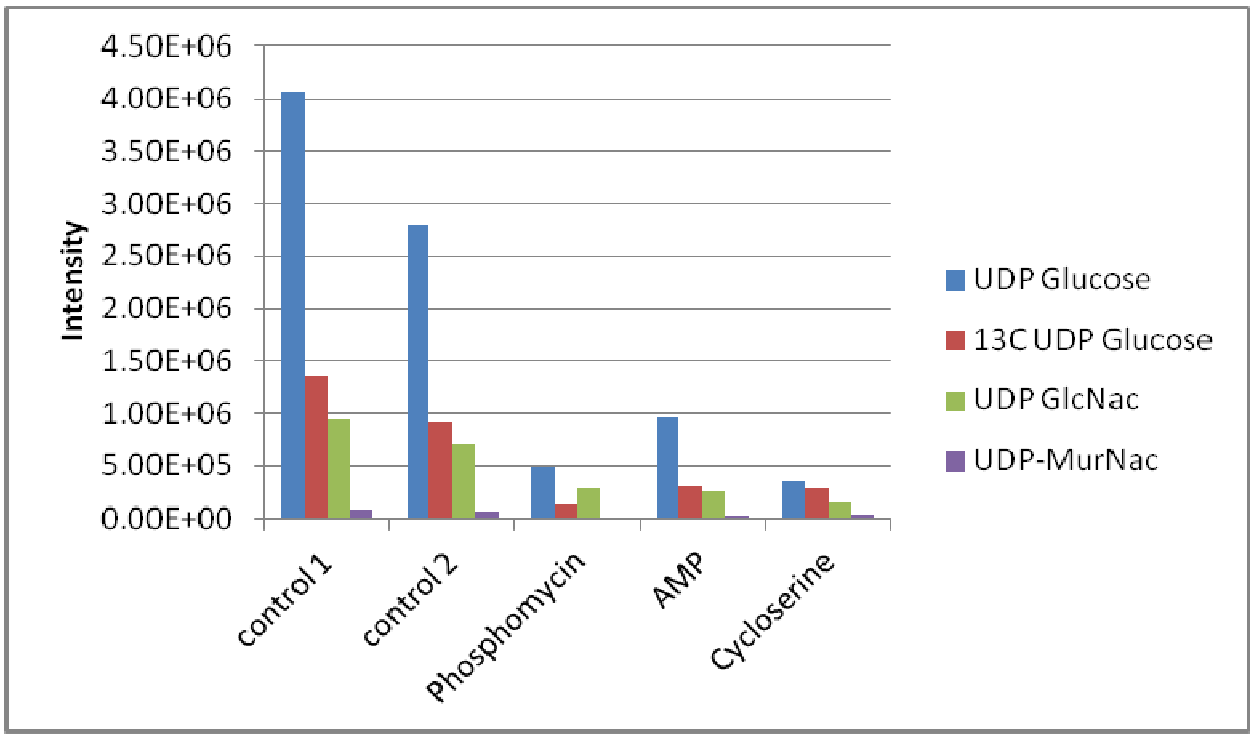
^{13}C UDP Glucose MS/MS analysis:

Mass (amu)	Fragmet mass	Parameter	Current value	New value	Intensity(cps)
567.814	138.875	DP	-45	-50	85716
567.814	158.790	EP	-10	-11.5	68849
567.814	240.950	CEP	-32.6	-22.0	83906

Table 3:UDP Samples with Antibiotics:

Sample	13C UDP-Glucose 567.3/385	UDP-GluNac 605.8/385	UDP-MurNac 677.9/385
Control-1	1.36E+06	9.51E+05	9.54E+04
Control -2(2ndExtraction)	9.14E+05	6.93E+05	6.77E+04
Phosphomycin	1.40E+05	2.89E+05	3.99E+04
Ampicillin	3.00E+05	2.52E+05	2.06E+04
Cycloserine	2.80E+05	1.49E+05	4.01E+04

figure: 11



Measured levels of ^{13}C internal standard from E.coli extracts in the absence and presence of 8× MIC for Phosphomycin, Ampicilin and Cycloserine.